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Molecular and functional characterization of an amphibian urea transporter

Cécile Couriaud ^a, Christine Leroy ^a, Matthieu Simon ^a, Claudia Silberstein ^b,
Pascal Bailly ^c, Pierre Ripoché ^a, Germain Rousselet ^{a,*}

^a *Service de Biologie Cellulaire, Bât. 532, CEA/Saclay, 91191 Gif-sur-Yvette, France*

^b *Departamento de Fisiología, Facultad de Medicina, Buenos Aires, Argentina*

^c *GIP-Institut National de la Transfusion Sanguine, INSERM U76, Paris, France*

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Abstract

We report the characterization of a frog (*Rana esculenta*) urea transporter (fUT). The cloned cDNA is 1.4 kb long and contains a putative open reading frame of 1203 bp. In frog urinary bladder, the gene is expressed as two mRNAs of 4.3 and 1.6 kb. The fUT protein is 63.1 and 56.3% identical to rat UT-A2 and UT-B1, respectively. The internal duplication of UT-A2 and UT-B, as well as the double LP box urea transporter signature sequence were found in this amphibian urea transporter. When expressed in *Xenopus* oocytes, fUT induced a 10-fold increase in urea permeability, which was blocked by both phloretin and mercurial reagents. The fUT protein did not transport thiourea, but the fUT-mediated urea transport was strongly inhibited by this compound. Thus, this amphibian urea transporter displays transport characteristics in between those of UT-A2 and UT-B. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Urea transporter; Phloretin; Thiourea; *Rana esculenta*

1. Introduction

In mammals, as well as in other terrestrial animals, urea is used to raise the osmolarity of the milieu intérieur, either throughout the body as in amphibians, or in specialized organs, as in the mammalian kidney. This allows to retain water inside the body and to avoid dehydration. In both cases, the water balance is maintained by the hormonal regulation of water and urea permeabilities of specialized epithelia

(the mammalian collecting ducts or the amphibian urinary bladder). This control is primarily exerted by vasopressin in mammals and vasotocin in amphibians (see [1] for a review).

Facilitated urea transporters have been cloned first in mammals [2,3]. In the terminal inner medullary collecting duct, the UT-A gene (according to the nomenclature proposed in [4]) is expressed at least as a 4.1 kb mRNA, encoding a 100 kDa protein called UT-A1 [5], localized to the apical membrane of principal cells [6]. The same gene is transcribed as a 2.9 kb mRNA encoding a 45 kDa protein called UT-A2 in epithelial cells from the descending thin limb of the loops of Henle [6,7], where it is probably involved in the recycling of urea to the deepest parts of the medulla in order to avoid a dissipation of the

* Corresponding author. Fax: +33 (1) 69 08 80 46;
E-mail: rousselet@dsvidf.cea.fr

osmotic gradient. Two other transcripts (UT-A3 and UT-A4), that encode different proteins, have recently been characterized [8]. Another urea transporter called UT-B [9] is expressed in the vasa recta of kidney [10] and in other tissues, such as red blood cells [11] and brain [12–14]. The UT-A2 and UT-B1 transporters are 65% identical.

The urea and water permeabilities of the apical membrane of principal cells from kidney collecting duct are controlled by vasopressin. A mechanism of vesicle trafficking regulation has been demonstrated for the vasopressin-regulated water channel, AQP2 [15–17], some 20 years after having been proposed in a model tissue: the amphibian urinary bladder [18,19]. In cells from the amphibian urinary bladder epithelium, vesicles called aggregophores are believed to carry water channels. The comparable and rapid time-courses of activation for water and urea permeability stimulations [20] suggested that urea transporters could be regulated through a similar mechanism. In mammalian kidney, it has first been suggested that AQP2 and UT-A1 might be carried by the same vesicles [6]. However, subsequent studies have shown that a trafficking mechanism is probably not involved in the regulation of urea permeability by AVP [21]. Water and urea permeabilities are actually separately regulated under some experimental conditions, both in mammals [22] and in amphibians [23].

In order to obtain molecular tools to investigate the cellular mechanisms of urea transport regulation in vasopressin-sensitive epithelia, we have cloned by homology to mammalian urea transporters a functional frog urinary bladder urea transporter (fUT).

2. Materials and methods

2.1. Molecular biology

All molecular biology experiments were performed according to standard protocols [24]. Enzymes were from New England Biolabs (Beverly, MA, USA), unless otherwise indicated. A frog (*Rana esculenta*) urinary bladder cDNA library was constructed in SWAJ-2 (Clontech, Palo Alto, CA, USA) from frog bladder polyA⁺ RNA. This library (2.4×10^6 independent clones), which was previously used to

clone the frog AQP1 [25], was screened at a moderate stringency (final wash: $0.5 \times \text{SSC}$, 0.1% SDS, 65°C for 30 min) with a mixture of rbUT-A2 (kindly provided by M.A. Hediger, Boston, MA, USA) and hUT-B1 cDNA probes labelled with [³²P]dCTP by random priming (Boehringer Mannheim France, Meylan, France). Three positive clones (#1, #4 and #5) were re-screened to homogeneity and their respective sizes were determined by PCR with SWAJ-2 specific primers. The cDNAs were then sub-cloned as *Eco*RI fragments (two fragments for clones #1 and #5 and one for clone #4) into pBluescript II SK– and a short sequence was determined on both sides. Two clones (#1 and #5) were found to be identical and only the longest (#5) was further analyzed. The sequence of clones #4 and #5 was determined on both strands by the dideoxy chain termination method, using restriction fragments and synthetic oligonucleotides when necessary. The sequence of clone #4 did not contain any open reading frame and was not homologous to any sequence in the nucleotide sequence libraries. This clone was not further analyzed. The sequence of clone #5 (fUT) was analyzed with the DNA Strider program [26] and the ExPasy resources of the University of Geneva (<http://expasy.hcuge.ch>).

2.2. Functional studies

The full length fUT coding sequence was reconstructed from the two *Eco*RI fragments into the pT7TS plasmid, a derivative of pGEM4Z containing the 5' and 3' untranslated sequences of the *Xenopus* β -globin gene (a kind gift of Dr P. Krieg, Austin, TX, USA). The pT7TSfUT plasmid was linearized and transcribed in vitro with the Stratagene in vitro transcription kit. The amount and quality of RNA were checked by spectrophotometry at 260 nm and agarose gel electrophoresis, respectively. *Xenopus laevis* oocytes were prepared as previously described [27] and micro-injected with 10 ng (50 nl) cRNA per oocyte. They were then incubated for 2–3 days in Barth's medium (in mM: 88 NaCl, 1 KCl, 0.8 MgSO₄, 0.3 Ca(NO₃)₂, 0.4 CaCl₂, 2.4 NaHCO₃, 7.5 Tris-HCl pH 7.6) at 18°C. Uptake experiments ([¹⁴C]urea, 8 $\mu\text{Ci/ml}$, 140 μM or [¹⁴C]thiourea, 8 $\mu\text{Ci/ml}$, 140 μM) were performed as previously described [13,27].

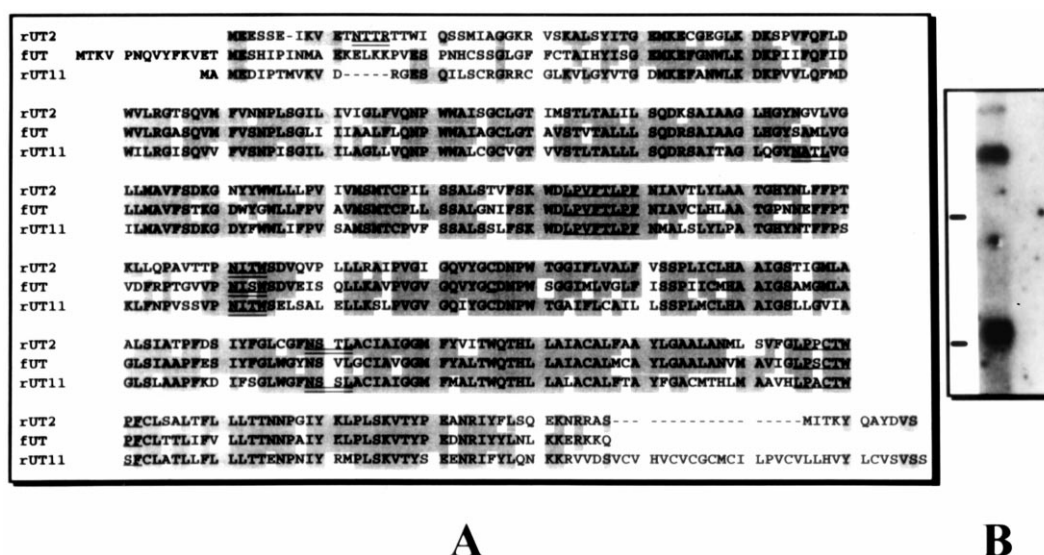


Fig. 1. Structure of fUT. A: Sequence alignment of fUT and other members of the urea transporter family. Conserved residues are in shaded boxes. The LP box is underlined and putative *N*-glycosylation sites are double-underlined. The complete nucleotide sequence has been deposited to the EMBL nucleotide sequence library under accession number Y12784. B: Transcription profile of fUT in the frog urinary bladder. The ribosomal RNAs are indicated on the left.

2.3. Northern blotting

Total RNA was prepared from frog bladder with the RANow mixture (Biogentex), according to the manufacturer's instructions. RNA samples (20 µg) were resolved by agarose electrophoresis in the presence of formamide and formaldehyde, blotted to a nitrocellulose membrane (BAS85, Schleicher and Schuell, Dassel, Germany) and hybridized overnight with a [³²P]dCTP-labelled fUT probe (random priming kit from Boehringer Mannheim France, Meylan, France) in standard hybridization buffer containing 50% formamide at 42°C. The blots were then washed in increasingly stringent buffers (last wash: 0.1×SSC, 0.1% SDS, 65°C) and exposed to X-ray films (X-omat, Kodak, New Haven, CT, USA) for 5 days.

3. Results

Screening of a *R. esculenta* urinary bladder cDNA library with a mixture of UT-A2 and UT-B probes is described in Section 2. A cDNA of 1.4 kb was obtained, containing an open reading frame of 1203 bp. The predicted sequence of the protein encoded by the cloned cDNA is presented in Fig. 1A. The fUT pro-

tein is 63.1 and 56.3% identical to rat UT-A2 and UT-B, respectively. Analysis of the sequences of the cloned urea transporters with the Clustal multi-alignment software did not allow to assign fUT neither to the UT-A nor to the UT-B sub-group (trichotomy UT-A/UT-B/fUT, data not shown). There is only one putative *N*-glycosylation site in the middle of the putative extracellular loop, suggesting that the other glycosylation sites found in mammalian urea transporters are probably not glycosylated in vivo.

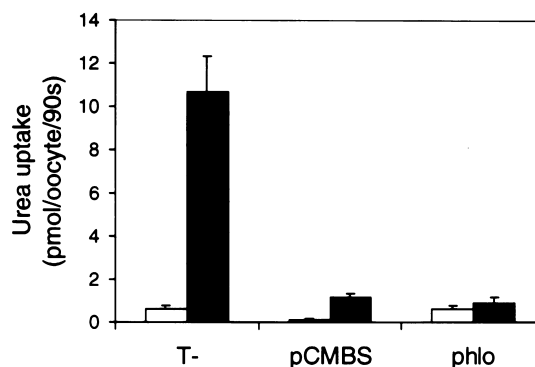


Fig. 2. Pharmacological analysis of fUT-mediated urea transport. Uptake at 90 s of [¹⁴C]urea into *Xenopus* oocytes injected with (closed bars) or without (open bars) 50 nl (10 ng) of fUT cRNA. Phloretin (phlo) and pCMBS (pCMBS) were at 0.5 mM and pre-incubation was for 20 and 10 min, respectively. Data are presented as mean ± S.E.M. of five oocytes.

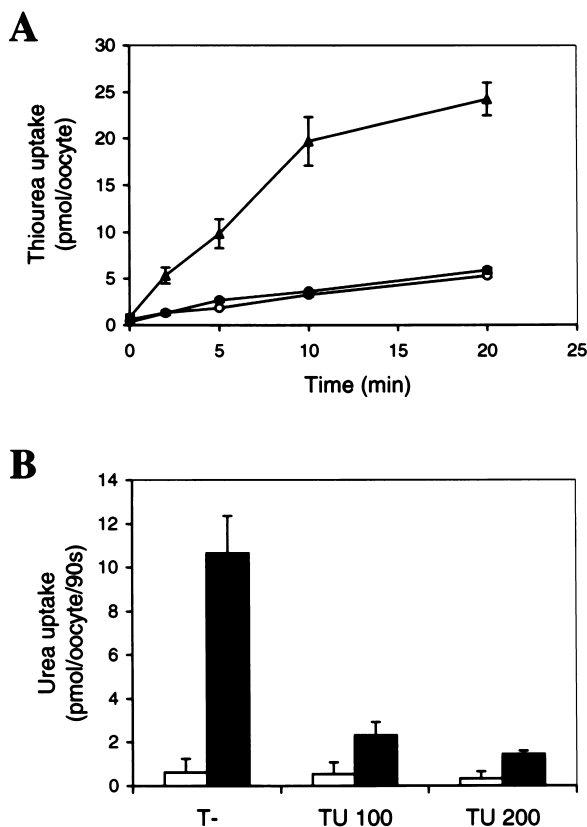


Fig. 3. Analysis of thiourea handling by fUT. A: Time-course of [^{14}C]thiourea uptake into *Xenopus* oocytes injected with 50 nl (10 ng) of fUT (closed circles) or rUT-B (closed triangles) cRNA or non-injected (open circles). Data are presented as mean \pm S.E.M. of five oocytes. B: Inhibition of urea transport by thiourea at 100 mM (TU100) or 200 mM (TU200). Urea transport was analyzed at 90 s under the same conditions as in Fig. 1. Data are presented as mean \pm S.E.M. of five oocytes.

No consensus phosphorylation site was detected. The double LP box, a signature sequence of urea transporters [28], is strictly conserved in this amphibian sequence. The gene is transcribed as two mRNAs of 4.3 and 1.6 kb in the amphibian urinary bladder (Fig. 1B). It is not known at this point whether these two mRNAs encode different proteins, as in UT-A for instance, or the same protein, as in hUT-B.

When expressed in *Xenopus* oocytes, the fUT protein induced a 10-fold increase in urea permeability (Fig. 2). Oocytes injected with the antisense cRNA had a urea permeability not significantly different from control oocytes (data not shown). The fUT-mediated urea uptake was fully inhibited by both

0.5 mM phloretin and 0.5 mM of the mercurial reagent *para*-chloromercuribenzenesulfonate (pCMBS) (Fig. 2). These pharmacological properties are similar to those of UT-B [27]. Although the fUT protein did not seem to open a detectable pathway for thiourea, the urea transport through fUT was very efficiently inhibited by this compound (Fig. 3). These transport characteristics are similar to those found for UT-A2 [27]. Altogether, these data demonstrate that fUT is an amphibian urea transporter with structural and functional characteristics in between those of UT-A2 and UT-B.

4. Discussion

The amphibian urinary bladder has for several years been an extensively worked model tissue for vasopressin-regulated epithelia. This model allowed for the establishment of paradigms in the regulation of water and urea transport by this hormone. However, it has greatly suffered from the difficulties encountered when applying standard molecular biology techniques. We have already reported the cloning of an aquaporin from frog urinary bladder [25], as well as its localization [29]. We now report the cloning of an urea transporter from frog urinary bladder, extending the range of molecules amenable to experimental analysis. This approach already allows to conclude that at least one facilitated urea transporter is expressed in the amphibian urinary bladder, confirming that this tissue could represent a good working model of vasopressin-sensitive epithelia, including at the molecular level.

Our results extend the numbers of facilitated urea transporter sequences available for analysis. The sequence identities between these urea transporters are surprisingly high. This shows that the selection pressure must be high and might be taken as an indication of strong structure function relationships in this family of membrane transporters. In this regard, the capacity of the UT-B family of transporters to transport thiourea, whereas neither fUT nor UT-A2 are permeant to this solute, might represent an indication of structural differences between two families of urea transporters. On the other hand, it is of note that none of the living organisms for which the genome has been fully sequenced, including *Caenorhab-*

ditis elegans, appears to possess homologous facilitated urea transporters. The most ancestral UT has recently been cloned in the elasmobranch *Squalus acanthia* [30]. As *C. elegans* has been shown to be ammoniotelic [31], it would be interesting to test whether there is a link in evolution between excretion of urea as a nitrogen waste product and expression of UT-related genes.

It is not clear yet whether fUT, or putative other isoforms encoded by the fUT gene, are regulated by vasopressin in vivo. The overall sequence identity of the fUT protein to either the UT-A or the UT-B isoform of mammalian urea transporters is similar (~65% versus ~60%, respectively). Furthermore, fUT does not contain the putative UT-A1 phosphorylation sites that are believed to be important in the regulation of UT-A1 by vasopressin. The functional profile of fUT expressed in *Xenopus* oocytes is on the one hand similar to the one observed for UT-B, as it is fully inhibited by pCMBS and thiourea. But on the other hand, like UT-A2, fUT does not transport thiourea. Therefore, it seems difficult at this point to ascribe fUT to either one of the UT-A or UT-B groups. An intriguing possibility, raised by the fact that we screened the cDNA library with a mixture of UT-A and UT-B without obtaining any other clone than fUT, is that amphibians may perhaps have only one urea transporter assuming the functions of both UT-A and UT-B.

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